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Validation of the OECD Reproduction Test Guideline with the New Zealand mudsnail *Potamopyrgus antipodarum* using trenbolone and prochloraz

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Abstract

The Organisation for Economic Cooperation and Development (OECD) provides several standard test methods for the environmental risk assessment of chemicals, mainly using primary producers, arthropods and fish. In April 2016, two new test guidelines with two mollusc species were approved by OECD member countries. One of the test guidelines focuses on a 28-day reproduction test with parthenogenetically reproducing New Zealand mudsnails *Potamopyrgus antipodarum*. The main endpoint of the test is reproduction, which is reflected by the embryo number in the brood pouch per female. The development of a new OECD test guideline involves several phases including validation studies such as ring tests to demonstrate the robustness of the proposed test design and the reproducibility of the test results. Therefore, a ring test of the reproduction test with *P. antipodarum* including eight laboratories and the test substances trenbolone and prochloraz was conducted. Results indicated that trenbolone did not have an effect on the reproduction of the snails in all participating laboratories in the tested concentration range. For prochloraz, the average EC_{10}, and NOEC values for reproduction (with coefficient of variation) were 24.1 µg/L (61.3%) and 30.5 µg/L (26.7%), respectively. This ring test demonstrates the robustness and the inter-laboratory reproducibility of the reproduction test with *P. antipodarum* and shows that it is a well-suited tool for the chronic aquatic risk assessment of chemicals.

Keywords

Test development, mollusc, gastropod, endocrine disruption, toxicity, fecundity

1 Introduction

Ecosystems may be contaminated by a wide range of xenobiotic chemicals that are capable of modulating or disrupting the endocrine system of organisms. In the last decades, these endocrine disrupting chemicals (EDCs) received considerable attention due to their potential to affect the reproductive success of animals even at low concentrations (Diamanti-Kandarakis et al. 2009; Gore et al. 2015; Vos et al. 2000).

The rising awareness of the potential impacts of EDCs motivated the Organisation for Economic Cooperation and Development (OECD) to compile the Conceptual Framework for Testing and Assessment of EDCs. Therein, different *in silico*, *in vitro* and *in vivo* methods are categorised in five levels with increasing biological complexity (OECD 2012a). This framework lists standardised tests methods, which are available or should be included in the OECD test guideline programme. Currently, the list of OECD test guidelines for the assessment of EDCs is dominated by vertebrates and arthropods. Invertebrates, although representing 95% of the animal kingdom, are still underrepresented in the OECD test guideline programme (Matthiessen 2008). More recently, the OECD supports the development of new invertebrate test guidelines (Gourmelon and Ahtiainen 2007). These tests belong to levels 4 and 5 of the Conceptual Framework, but involve only tests with apical endpoints (e.g. reproduction). Therefore, the tests are able to assess adverse effects on reproduction which is under endocrine control, although an altered reproductive output does not necessarily indicate an endocrine mechanism of the test compound.

The mollusc phylum represents a particularly promising taxon for the risk assessment of chemicals because these invertebrates are sensitive to a wide number of toxicants including EDCs (Matthiessen and Gibbs 1998; Oehlmann et al. 2007). Molluscs are also abundant found in many ecosystems and are highly ecologically and economically important (Duft et al. 2007). With about 130,000 known species, it represents the second largest phylum next to the arthropods (Gruner 1993). Inclusion of molluscs in the OECD test guideline programme for the risk assessment of chemicals would provide a more representative coverage of the animal kingdom. In a detailed review paper on Molluscs Life-Cycle Toxicity Testing (OECD 2010a), three candidate species including possible test designs for the development of a standardised chronic toxicity test were identified. One of the proposed species was the New Zealand mudsnail *Potamopyrgus antipodarum*. This species is known to be sensitive to a wide range of chemicals identified as EDCs in vertebrates and also to reproductive toxicants such as cadmium (Geiß et al. 2016; Gust et al. 2010; Jobling et al. 2003; Ruppert et al. 2016a; Sieratowicz et al. 2011).

The development of a new OECD test guideline involves a number of successive steps. Before a test guideline can be submitted to the OECD, several validation stages have to be performed, in form of ring tests. The objective of a ring test is to demonstrate the robustness of the proposed test design and to investigate the reproducibility of test results among several laboratories (OECD 2005).

The present study shows the results of such a ring test for the validation of the reproduction test with *P. antipodarum*. Eight laboratories participated in this ring test coming from academia, government and industry. Trenbolone and prochloraz were chosen as test chemicals, as both are known endocrine disrupters in vertebrates. The assumed main mode of action of prochloraz based on its effects in fish is the inhibition of aromatase, whereas trenbolone is a synthetic non-aromatizable androgenic steroid (Ankley et al. 2003; Matthiessen and Weltje 2015; Wilson et al. 2002). Both substances have already been used in validation studies for other OECD test

guidelines (OECD 2006a; OECD 2006b; OECD 2011). Our study aimed to investigate the inter-laboratory robustness and reproducibility of the proposed test design as well as to provide the first study of the effects, if any, of trenbolone and prochloraz on the reproduction of *P. antipodarum*. To this date, the reproduction test with *P. antipodarum* has been officially approved by the national coordinators of the OECD member countries as a test guideline in April 2016.

2 Materials and Methods

2.1 Test organism

The New Zealand mudsnail *Potamopyrgus antipodarum* (phylum Mollusca, class Gastropoda, family Hydrobiidae) was introduced to Europe and other parts of the world over 150 years ago, mainly with the ballast water of ships (Ponder 1988; Städler et al. 2005). The snails can be found in freshwater ecosystems and in estuaries up to a salinity of 15‰. Mudsnails prefer living on soft sediment and their natural diet are detritus, algae and bacteria (Duft et al. 2007; Jacobsen and Forbes 1997). Three clonal genotypes were identified in Europe by Hauser et al. (1992): clone A is found in freshwater ecosystems, clone B prefers estuaries and clone C is widespread in the United Kingdom (Städler et al. 2005).

In contrast to the all-parthenogenetic invasive populations in Europe (Robson 1923; Wallace 1979), *P. antipodarum* populations in its native New Zealand often feature coexistence of parthenogenetic individuals with obligately sexual males and females (Lively 1987). Both parthenogenetic and sexual female *P. antipodarum* reproduce ovoviviparously (Winterbourn 1970), which takes place throughout the year (Sieratowicz et al. 2011). The pallial oviduct is transformed to a brood pouch, where the developing embryos are located until the juvenile snails hatch (Fretter and Graham 1994).

2.2 Principle of the reproduction test

Adult laboratory-cultured parthenogenetic female *P. antipodarum* in a defined size class (3.5 - 4.5 mm) are exposed to a concentration range of the test substance, a negative (only test water) control and, if needed, a solvent control group over 28 days. The endpoint of the test is the reproduction of the mudsnails, which is reflected by the embryo numbers in the brood pouch per female at the end of the exposure period. However, mortality is assessed as well. The test chemical is added into reconstituted water and six snails are subsequently introduced per test beaker. Six replicates are used for each treatment group. The reproduction test is carried out at a water temperature of 16 ± 1°C and a light: dark regime of 16:8 h with a light intensity of 500 ± 100 lx.

2.3 Experimental conditions

The ring test was conducted in 2014. For the presentation of data, the participating laboratories are anonymised and laboratory codes were used instead of names. Snails used for this ring test were obtained from the laboratory culture at Goethe University Frankfurt am Main, Germany, which was built up with specimens collected in August 2011 from a small creek named Lumda near Rabenau, Germany. Each participating laboratory received 500 snails, except for laboratory 3P. This laboratory used specimens of *P. antipodarum* sourced from their own laboratory culture, which was built up with specimen from lake Te Anau in Fiordland, New Zealand. These snails were acclimatized for 28 days to the reconstituted water used in this ring test because these snails are normally cultured with carbon-filtered tap water. To ensure recovery from shipping stress after arrival in the participating laboratories, snails were acclimated to the laboratory conditions for at least 13 days before testing commenced.

The experimental conditions are summarized in Table 1. All laboratories were provided with a draft Test Guideline for the implementation of the reproduction test with *P. antipodarum*. Tests were carried out in a semi-static test design with water renewal three times per week for all exposure and control groups. Also laboratories were provided with the test chemicals from a

single batch prepared by Goethe University Frankfurt am Main. Trenbolone (CAS-No.: 10161-33-8, Sigma-Aldrich®, Germany) was tested at nominal concentrations of 10, 30, 100, 300 and 1000 ng/L. The nominal concentrations of the fungicide prochloraz (CAS-No.: 67747-09-5, Sigma-Aldrich®, Germany) were 3.2, 10, 32, 100 and 320 µg/L. For both substances dimethyl sulfoxide (DMSO; CAS: 67-68-5) was used as solvent at a concentration of 10 µL/L. Therefore, an additional solvent control group with the identical DMSO concentration as in the exposure groups was required. As both chemicals were tested at the same time, only one negative and one solvent control group were used.

Table 1: Summary of experimental conditions (modified after Ruppert et al. (2016b).

Test duration	28 days
Test water	400 mL reconstituted water (0.3 g TropicMarin® sea salt and 0.18 g $NaHCO_3$ per 1 litre deionised water)
Water quality requirements	pH: 7.5 - 8.5; conductivity: 770 ± 100 µS/cm; oxygen saturation: > 60% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids, change of test beakers once per week
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lx
Water sampling	Pooled samples were taken from all tested concentrations (trenbolone and prochloraz) and solvent control over four renewal intervals
Photoperiod	16:8 h (light: dark)
Food source	Finely ground Tetraphyll®
Feeding	0.2 ± 0.05 mg per snail and day
Snails origin	Laboratory culture from Goethe University; own culture in laboratory 3P
Test snails size	3.5 - 4.5 mm
Snail density	6 snails per test beaker (6 replicates per treatment group)
Test endpoints	Reproduction, mortality

Mudsnails were exposed in closable 500 mL glass beakers filled with 400 mL reconstituted water (for medium composition see Table 1). The conductivity of the test medium should be achieved and kept at 770 ± 100 µS/cm and pH should be adjusted to 8.0 ± 0.5 with NaOH and HCl. Snails were fed with finely ground Tetraphyll® (0.25 mg per snail per day, Tetra GmbH, Melle, Germany) after each medium renewal. Test water was gently aerated through glass Pasteur pipettes connected to an air tubing system. The participating laboratories were asked to

replace test vessels once per week. Water quality parameters (pH, conductivity, temperature, oxygen saturation) were measured and recorded three times per week immediately before water renewal in one replicate per treatment group.

After 28 days exposure, mudsnails were quick-frozen in liquid nitrogen or sacrificed at -20°C in the freezer. Shell length was measured by means of a stereomicroscope, snails were dissected and the number of embryos in the brood pouch per female was recorded.

2.4 Analytical measurement

Analytical determinations of trenbolone and prochloraz in water samples were conducted by the University of Southern Denmark, Odense, Denmark. Water samples from all treatment groups of trenbolone and prochloraz, including the solvent control group, were taken over four renewal intervals. Therefore, samples of freshly prepared and of two- or three-day old medium were taken every week for chemical analyses. Samples acquired from old medium were pooled from all replicates per treatment group. Samples were stored at -20°C in HDPE-bottles until shipment to the University of Southern Denmark. Nominal concentrations of trenbolone and prochloraz were quantified using liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS, Agilent 1200 series triple quadrupole). The limits of detection (LOD) for trenbolone and prochloraz were 0.39 ng/L and 1.56 µg/L, respectively. Trenbolone samples were extracted on solid-phase columns with methyl-testosterone as internal standard before analysis. Prochloraz was directly measured from filtered samples. According to annex 6 of the OECD guideline 211 (OECD 2012b), time-weighted mean (TWM) concentrations of the chemicals were calculated for each laboratory.

2.5 Biological raw data analysis

Biological raw data (mortality, shell length and embryo numbers) were recorded by the participating laboratories using a spreadsheet previously provided by the Goethe University

Frankfurt am Main, Germany. Statistical evaluations were performed using GraphPad Prism® (Version 5.03, GraphPad Software Inc., San Diego, USA) and Microsoft Excel® (Microsoft Corporation, Redmond, USA). The Fisher's exact test was used to test for differences in mortality between treatment and control groups. For the embryo numbers, arithmetic mean values of each replicate per treatment group were calculated and these were used for statistical analysis. If negative and solvent controls did not differ significantly by using the unpaired t-test, both were merged to one control group (Green and Wheeler 2013). Effect concentrations were calculated by one-way analysis of variances (ANOVA) followed by Dunnett's multiple comparison test to find statistical differences compared to the control group. The 10% and 50% effect concentrations (EC_{10} and EC_{50}) for each laboratory were determined by using a LogNorm or Weibull non-linear regression model (Christensen et al. 2009). The best-fitting model was chosen, i.e. the lowest r^2.

2.5 Validity criteria

Based on available test guidelines for utilising freshwater invertebrates (OECD 2004; OECD 2012b) and on the results of earlier ring tests (Ruppert et al. 2016b), the following validity criteria were required to be fulfilled throughout each test:

- mortality in controls should not exceed 20%;
- mean embryo numbers per snail in the control should be ≥ 5;
- dissolved oxygen should be at least 60% of the air saturation value (ASV); and
- water temperature should be 16 ± 1°C.

3 Results

3.1 Water quality parameters and compliance with validity criteria

All participating laboratories achieved the recommended water quality parameters (Table 2). The physico-chemical validity criteria (temperature and oxygen saturation) were met in all

laboratories in which these data were obtained. The temperature ranged between 15.7°C and 16.5°C and the oxygen saturation ranged between 94.4% and 99.6%.

Table 2: Mean (with standard deviation; SD) water quality parameters from all participating laboratories. n.r.: not received.

	pH		Conductivity [µS/cm]		Temperature [°C]		O_2 saturation [%]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lab. 3A	8.28	0.690	791	39.5	16.2	0.610	96.1	3.06
Lab. 3D	8.26	0.676	750	23.2	15.9	0.438	99.3	1.41
Lab. 3H	8.33	0.676	725	37.3	15.9	0.214	94.4	4.20
Lab. 3L	8.44	0.673	718	23.6	16.5	0.340	98.4	2.31
Lab. 3M	8.11	0.650	751	30.2	16.4	0.851	99.4	0.851
Lab. 3N	8.24	0.660	722	18.6	15.8	0.342	96.6	6.95
Lab. 3O	8.16	0.682	818	15.1	16.3	0.523	99.6	1.75
Lab. 3P	7.58	0.737	n.r.	n.r.	15.7	0.572	n.r.	n.r.

Only two laboratories did not meet the biological validity criteria. Laboratory 3H observed a high mortality of the snails in control and exposure groups (Fig. 1). Mortalities in the negative and the solvent controls were 22.2% and 30.6%, respectively, and ranged between 11.1% and 36.1% in the trenbolone exposure groups. A much lower mortality rate of 2.78% occurred at the two highest tested concentrations of prochloraz in this laboratory.

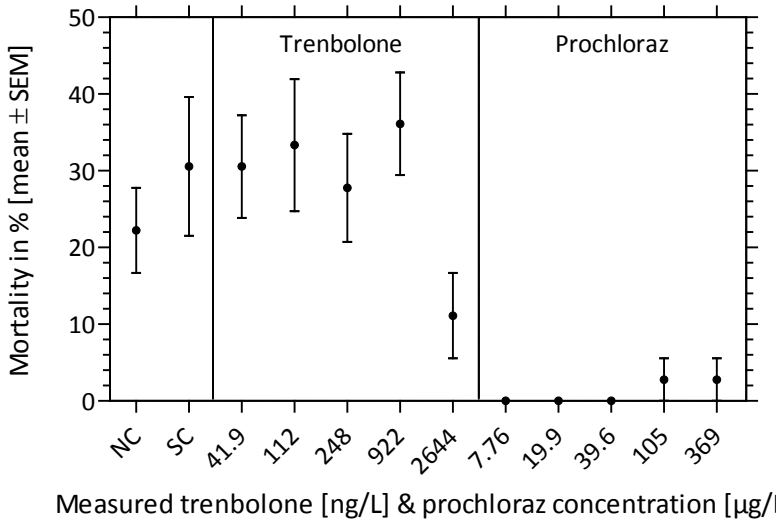

Figure 1: Mortality (mean with standard error; SEM; n = 6) of *Potamopyrgus antipodarum* after 28 days exposure to time-weighted means of measured trenbolone and prochloraz concentrations at laboratory 3H.

The mean embryo numbers in the controls were above 5 for all laboratories, except for laboratory 3P. Here, the mean embryo numbers in controls were 1.08 (data not shown). As the reproduction tests from laboratories 3H and 3P were not valid, test results from these laboratories were not considered in the following evaluation. Data on the actual exposure concentrations and the reproduction data from both laboratories can be found in the Supplemental Information.

3.2 Actual exposure concentrations

Tables 3 and 4 summarize the calculated TWM concentrations of trenbolone and prochloraz from all participating laboratories with valid test results. More detailed information on the actual exposure concentrations of each participating laboratory can be found in the Supplemental Information. For all laboratories except laboratory 3N, TWM concentrations were higher compared to nominal concentrations and varied between 50% and 627%. The measured trenbolone concentration in the solvent control group was below the LOD for all laboratories, except for samples from laboratory 3L and 3M. At laboratory 3M, trenbolone was detected in all control samples. Here, the calculated TWM concentration of trenbolone in the solvent control group was 14 ng/L. At laboratory 3L, trenbolone was detected in 5 out of 8 solvent control samples with concentrations ranging between 1.25 and 33.2 ng/L resulting in an arithmetic mean concentration of 9.31 ng/L.

Table 3: Time-weighted mean concentrations of trenbolone (in ng/L) in exposure media from all participating laboratories with valid test results. -: not detected; SC: solvent control.

Nominal concentrations [ng/L]	Time-weighted mean concentrations [ng/L]					
	Lab. 3A	Lab. 3D	Lab. 3L	Lab. 3M	Lab. 3N	Lab. 3O
SC	-	-	9.31[1]	14.0	-	-
10	13.3	19.7	31.6	27.4	8.92	16.7
30	34.4	55.1	75.5	50.2	14.9	38.4
100	132	350	217	173	60.9	170
300	372	1882	469	396	177	496
1000	1373	4763	3205	1335	1046	2043

[1]: arithmetic mean concentration

Prochloraz concentrations were also found to be above the nominal concentrations and varied between 118% and 981% of nominal concentrations. Measured concentrations in the solvent control group were below the LOD, except for those from laboratories 3D, 3L and 3N. At laboratory 3D, prochloraz was detected during the last two renewal intervals with a maximum concentration of 9.63 µg/L. At laboratory 3N, prochloraz was only detected in a single sample of old medium with a concentration of 1.20 µg/L. Prochloraz was found in all solvent control samples at laboratory 3L, which resulted in a TWM concentration of 9.98 µg/L. Because both chemicals were measured in the solvent control group of laboratory 3L, this test was classified as not valid and results were excluded from the evaluation of the embryo numbers. Furthermore, in this laboratory, the embryo numbers in the solvent control were significantly reduced compared to the negative control ($p = 0.0193$).

Table 4: Time-weighted mean concentrations of prochloraz (in µg/L) in exposure media from all participating laboratories with valid test results. -: not detected; SC: solvent control.

Nominal concentrations [µg/L]	Time-weighted mean concentrations [µg/L]					
	Lab. 3A	Lab. 3D	Lab. 3L	Lab. 3M	Lab. 3N	Lab. 3O
SC	-	4.04[1]	9.98	-	1.20[2]	-
3.2	10.3	31.4	13.5	22.7	10.4	11.9
10	27.3	58.2	24.6	32.9	23.0	21.3
32	51.4	52.8	42.5	58.3	40.4	40.0
100	229	266	160	305	194	183
320	183	529	379	626	468	489

[1]: arithmetic mean concentration; [2]: corresponds to a single contamination

In the OECD test guideline No. 211 (OECD 2012b), it is recommended that if measured concentrations have been maintained within ± 20% of the nominals, then results can be based on nominal concentrations. As TWMs of measured concentrations for both chemicals deviated

289 by more than 20% from nominals for all laboratories, calculations of effect concentrations were
290 based on the TWMs of measured concentrations.
291
292 **3.3 Biological responses**
293 The results of laboratories (3H, 3L, 3P) with non-valid test results are depicted in the
294 Supplemental Information.
295
296 **3.3.1 Effects of trenbolone on *Potamopyrgus antipodarum***
297 No mortality occurred in the negative and solvent control groups of laboratories reporting valid
298 test results. In laboratories 3A, 3D, 3M and 3O no mortality occurred in any of the exposure
299 groups. In laboratory 3N, a mortality of 2.78% occurred at the test concentrations of 14.9 ng/L
300 and 60.9 ng/L, respectively.
301 The mean embryo numbers in the merged negative and solvent control group ranged between
302 13.3 and 18.6 in the five participating laboratories. None of the laboratories found a
303 concentration-dependent effect of trenbolone on the reproduction of *P. antipodarum* (Fig. 2).
304 Only laboratory 3A detected significant reductions of embryo numbers at the two lowest test
305 concentrations (13.3 ng/L and 34.4 ng/L; $p < 0.05$), which were not observed at higher
306 concentrations.

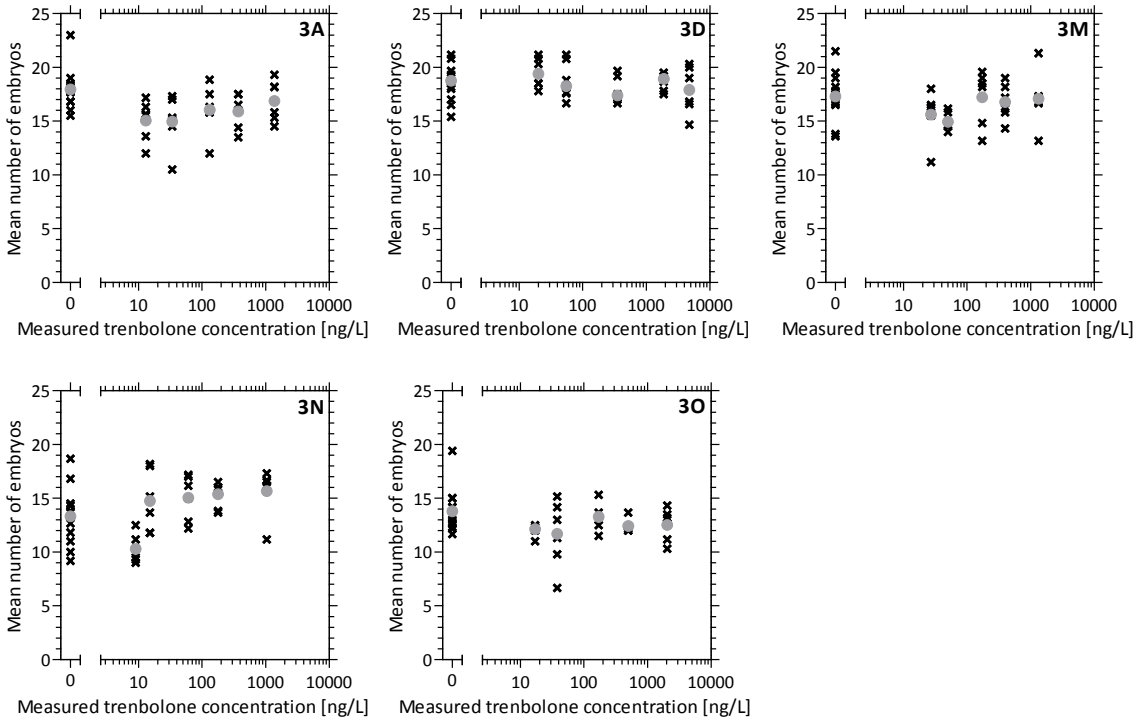

Figure 2: Mean embryo numbers of *Potamopyrgus antipodarum* after 28 days exposure to time-weighted means of measured trenbolone concentrations (in ng/L) in all participating laboratories with valid test results. Crosses depict the mean of each replicate and grey dots present the mean value of the treatment group. Number of replicates: 6 per exposure group, 12 for merged controls.

3.3.2 Effects of prochloraz on *Potamopyrgus antipodarum*

Only laboratory 3N observed mortalities at prochloraz concentrations of 10.4 µg/L and 194 µg/L. Compared to the control group, mortalities were significantly enhanced to 8.33% ($p = 0.035$) and 11.1% ($p = 0.011$), respectively (data not shown).

The reproduction of *P. antipodarum* was significantly ($p < 0.05$) impacted by prochloraz in all laboratories, with embryo numbers decreasing with increasing prochloraz concentrations. Figure 3 shows the concentration-response curves of the five partner laboratories reporting valid test results.

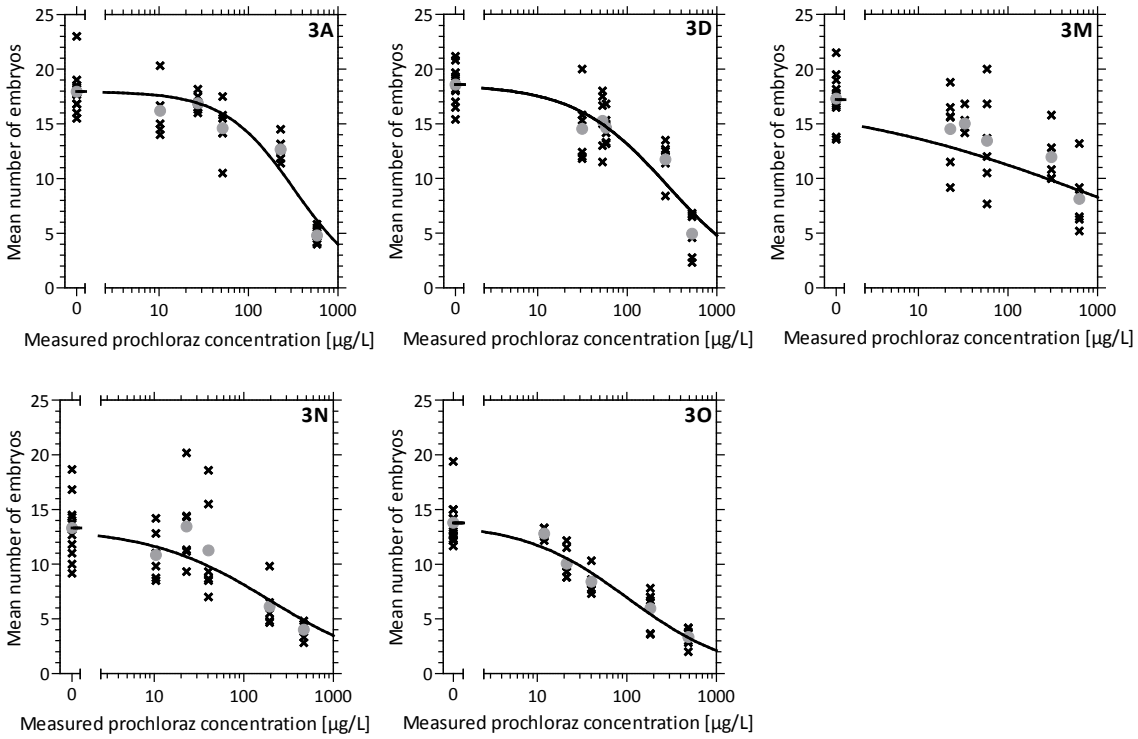

Figure 3: Mean embryo numbers of *Potamopyrgus antipodarum* after 28 days exposure to time-weighted means of measured prochloraz concentrations (in µg/L) in all participating laboratories with valid test results. Crosses depict the mean of each replicate and grey dots present the mean value of the treatment group. Number of replicates: 6 per exposure group, 12 for merged controls.

All laboratories found comparable effect concentrations including no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values (Table 5). The NOEC ranged from 21.3 to 40.4 µg/L with a 1.90-fold difference between the lowest and the highest effect concentration. The *P. antipodarum* used for testing in laboratory 3D showed the highest sensitivity towards an exposure of prochloraz. Here, already at the lowest test concentration of 31.4 µg/L, a significant ($p < 0.01$) reduction of embryo numbers was observed relative to the control group. The LOEC values of all laboratories are also in a comparable range between 31.4 and 194 µg/L. The good match of results is also reflected by the EC_x values as most of their 95%-confidence intervals overlap. The EC_{10} ranged between 6.04 µg/L and 45.4 µg/L and the EC_{50} from 103 µg/L to 763 µg/L.

Table 5: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals in brackets, NOEC and LOEC) for the total embryo numbers of *Potamopyrgus antipodarum* after 28 days exposure to time-weighted means of measured prochloraz concentrations (in µg/L) and the calculated average effect concentration (including coefficient of variation; %) from all valid tests.

[µg/L]	Lab. 3A	Lab. 3D	Lab. 3M	Lab. 3N	Lab. 3O	Mean effect concentration	Coefficient of variation
EC_{10}	45.4 (25.3 - 81.5)	24.9 (9.11 - 67.8)	15.6 (2.27 - 107)	28.3 (6.50 - 128)	6.04 (3.20 - 11.4)	24.1	61.3%
EC_{50}	327 (257 - 416)	285 (190 - 429)	763 (289 - 2015)	200 (116 - 346)	103 (75.3 - 140)	336	75.7%
NOEC	27.3	-	32.9	40.4	21.3	30.5	26.7%
LOEC	51.4	31.4	58.3	194	40.0	75.0	89.7%

The average effect concentrations (with coefficient of variation) for prochloraz of all valid tests are 24.1 µg/L (61.3%), 336 µg/L (75.7%), 30.5 µg/L (26.7%) and 75.0 µg/L (89.7%) for EC_{10}, EC_{50}, NOEC and LOEC, respectively (Table 5). The effect concentrations show a minimum of a 1.90-fold difference (NOECs) and a maximum of a 7.52-fold difference (EC_{10}).

4 Discussion

4.1 Effects of trenbolone

Snails exposed to trenbolone in the tested concentration range did not show a concentration-dependent effect on reproduction in any of the participating laboratories. This corresponds to the outcome of a study with the pondsnail *Lymnaea stagnalis* (Ducrot and Charles 2015). Here, two laboratories tested trenbolone in a concentration range between 9 ng/L and 394 ng/L in the reproduction test with *L. stagnalis* and did not observe any concentration-dependent change of fecundity. Whilst published toxicity data for other invertebrate species are lacking, these findings with molluscs differ from results of studies with other aquatic vertebrates. Holbech et al. (2006) used the fish sexual development test (FSDT) with the zebrafish *Danio rerio* to assess the toxicity of trenbolone-acetate and found a change in sex ratio on day 59 post-hatch to an all-male population at 9.7 ng/L and higher concentrations. The fecundity of the fathead minnow *Pimephales promelas* was significantly reduced at trenbolone concentrations of 27 ng/L and

362 above (Ankley et al. 2003). Olmstead et al. (2012) showed that the western clawed frog *Xenopus*
363 *tropicalis* is negatively affected by exposure to trenbolone during larval development and
364 demonstrated a shift in sex ratio towards males at 78 ng/L.
365 Ankley et al. (2003) investigated the binding affinity of trenbolone to the androgen receptor of
366 the fathead minnow in an *in vitro* binding assay and found that trenbolone had a higher binding
367 affinity for the receptor than testosterone. To date, no androgen receptor has been identified in
368 any mollusc species (McClellan-Green 2013). Despite the apparent absence of an androgen
369 receptor, the exposure to androgens causes the development of male sex organs (imposex) of
370 females in several gastropod species (Bettin et al. 1996; Janer et al. 2006b; Oehlmann et al.
371 2007). Janer et al. (2006a) demonstrated that androgens can be converted to dihydro-
372 testosterone in the gastropod *Marisa cornuarietis* and that this pathway is specifically inhibited
373 by organotin compounds. Previous studies have also shown significant reductions of embryo
374 numbers in *Potamopyrgus antipodarum* following exposure to methyl-testosterone in the lower
375 ng/L range (Duft et al. 2007). These conflicting findings for the two potent vertebrate androgen
376 receptor agonists trenbolone (reported here) and methyl-testosterone (Duft et al. 2007) could
377 be due to the differing biotransformation of the two compounds. Methyl-testosterone can be
378 aromatized to methyl-estradiol, whereas trenbolone can neither be aromatized nor transformed
379 to dihydro-testosterone (Baumann et al. 2014; Hornung et al. 2004; Wilson et al. 2002; Yarrow
380 et al. 2010). These previously described studies indicate that externally administrated androgens
381 can induce specific effects in molluscs which are under endocrine control. The results from the
382 present study using trenbolone show that the reproduction test with *P. antipodarum* did not
383 respond to this potent agonist of the androgen receptor of vertebrates in the tested concentration
384 range. Within the OECD Conceptual Framework for Testing and Assessment of EDCs, the
385 reproduction test with *P. antipodarum* belongs to level 4. Level 4 tests represent *in vivo* assays
386 providing data on adverse effects on endocrine-relevant endpoints such as development and
387 reproduction which may also be influenced by other modes of action (OECD 2012a). In

388 consequence, level 4 tests are limited for the identification of EDCs, because observed effects
389 are not necessarily endocrine-mediated. Therefore, the reproduction test with *P. antipodarum*
390 should not be treated as a surrogate of standard tests with vertebrates, but complements the
391 OECD test battery for the risk assessment of chemicals.
392
393 **4.2 Effects of prochloraz**
394 Prochloraz is an imidazole fungicide and registered for use for example on wheat, barley and
395 mushrooms (EFSA 2011). This fungicide acts via the inhibition of the cytochrome P450-
396 dependent 14α-demethylase (Henry and Sisler 1984), which plays a key role in the biosynthesis
397 of ergosterol as an essential constituent of fungal cell membranes. The functional group of
398 prochloraz interacts with the iron atom of cytochrome P450. As this binding is unspecific,
399 prochloraz and other imidazoles are also able to inhibit a broad spectrum of other cytochrome
400 P450-dependent enzymes. This inhibition extends to enzymes involved in the biosynthesis and
401 metabolism of steroids in several organisms (Vinggaard et al. 2006). As the mode of action of
402 prochloraz in snails is not known, the decrease of the embryo numbers in *P. antipodarum* that
403 we observed in our study may be caused by its interaction with cytochrome P450-dependent
404 monooxygenase pathways, including those involved in vertebrate steroid metabolism.
405 However, it cannot be excluded that the effect of prochloraz on the reproduction of
406 *P. antipodarum* could also be attributed to a general toxicity of the test substance.
407 The average effect concentrations for prochloraz (NOEC: 30.5 µg/L; LOEC: 75.0 µg/L; EC_{10}:
408 24.1 µg/L; EC_{50}: 336 µg/L) from our ring test with the mudsnail are in the range of effect data
409 of other test species. The reported NOEC for *Daphnia magna* in a 21-day reproduction test was
410 22.2 µg/L (EFSA 2011). For fish species, the obtained NOEC and LOEC values for the
411 endpoint sex ratio in the FSDT with *D. rerio* were 64 µg/L and 202 µg/L, respectively
412 (Kinnberg et al. 2007). The detected NOEC in a full life-cycle test with the fathead minnow
413 was 24.9 µg/L (EFSA 2011). Thorpe et al. (2011) reported a significantly lower proportion of

414 female zebrafish and fathead minnows at 100 µg/L and 320 µg/L, respectively. Experiments
415 performed by Zhang et al. (2008) with the Japanese medaka *Oryzias latipes* and prochloraz
416 showed a significant decrease in fecundity at 30 µg/L (LOEC).
417
418 **4.3 Reproducibility and robustness of the proposed test design**
419 All participating laboratories were able to perform the reproduction test with *P. antipodarum*,
420 independently from their level of experience in toxicity testing using a mollusc species. The
421 results of the reproduction tests with trenbolone and prochloraz showed a good match among
422 laboratories. The embryo numbers in the negative control groups were comparable among
423 partners and achieved coefficients of variation ranging between 5.40% and 18.7%. These values
424 fit well with the recommendations given in the OECD test guideline No. 211, the *D. magna*
425 reproduction test (OECD 2012b), where a coefficient of variation in controls of $\leq 25\%$ is
426 mentioned for a well-run test. Furthermore, for prochloraz, the participating laboratories
427 provided comparable effect concentrations in a narrow range. The inter-laboratory
428 reproducibility of the effects is expressed as the coefficients of variation and is acceptable when
429 comparing with other validation studies of chronic toxicity tests conducted with other
430 invertebrate species. A ring test study for the validation of the OECD test guideline No. 225
431 (OECD 2007), the chronic toxicity test with *Lumbriculus variegatus*, was performed with 14
432 laboratories and the test substance pentachlorophenol. For the endpoint reproduction (increase
433 in the number of worms), coefficients of variation were between 37.9% (EC_{50}) and 68.6%
434 (LOEC) and showed a maximum inter-laboratory factor of 23.8, which is higher compared to
435 the results reported here, with a maximum inter-laboratory factor of 7.52. In another validation
436 study, four laboratories performed a life-cycle test with the non-biting midge
437 *Chironomus riparius* and the substance pyriproxifen for the validation of the OECD test
438 guideline No. 233 (OECD 2010b). They found similar NOEC values for the endpoint fecundity
439 between 4 and 20 µg/L with an inter-laboratory factor and a coefficient of variation of 5 and

58.5%, respectively (OECD 2010c; Taenzler et al. 2007; Tassou and Schulz 2009). Ducrot et al. (2014) performed a ring test for the validation of the reproduction test with *L. stagnalis* including seven laboratories. Five tests achieved the validity criteria and found comparable effect concentrations of cadmium on reproduction. For the number of clutches, EC_{50} values ranged between 81.6 and 203 µg/L and the coefficient of variation was 44.2%.

In the present study, six out of eight laboratories fulfilled the given validity criteria, which demonstrates the robustness of the test design. Laboratory 3H exceeded the validity criterion for the maximum mortality of 20% in both control groups. The apparently high mortality rates in control and trenbolone exposure groups were probably caused by fungal growth during the reproduction test (see Fig. 1). The fungicide prochloraz prevented the growth of fungus and therefore reduced the mortality of snails in the exposure groups with prochloraz. Due to a lack of test vessels, laboratory 3H did not change the glass beakers once per week as foreseen in the draft Test Guideline of the ring test. Residual food in the test vessels has likely promoted the growth of fungus.

Laboratory 3P did not achieve the validity criterion for the minimum embryo number of 5 in the control groups. This laboratory was the only one to use snails derived from their own culture. Snails in laboratory 3P are normally cultured in carbon-filtered tap water, in contrast to the reconstituted water used for the culture of *P. antipodarum* at Goethe University. Even prior to the start of the test, the mean embryo number of 20 snails was examined and was 1.00, showing that the acclimation period to the test medium was probably too short.

5 Conclusions

In total, four validation studies of the reproduction test with *Potamopyrgus antipodarum* have been performed with 17 participating laboratories and six test compounds (Ruppert et al. 2016b). Over the course of these studies, the test design was optimised, e.g. using six replicates instead of four to increase the statistical power of the test. The robustness as well as the inter-

and intra-laboratory reproducibility have been demonstrated within the validation studies as laboratories reported comparable NOEC, LOEC, EC_{10} and EC_{50} values with mostly overlapping 95%-confidence intervals for EC_x values, even if difficult-to-handle substances, like tributyltin were chosen as test substance (Ruppert et al. 2016b).

After a second international commenting round by OECD member states, the guidelines of the reproduction test with *P. antipodarum* and the reproduction test with the pondsnail *Lymnaea stagnalis* were adopted by the national coordinators of the OECD member countries in April 2016. Both assays are the first invertebrate tests with aquatic non-arthropod species, to be successfully validated in the OECD Conceptual Framework for Endocrine Disrupters as level 4 assays (OECD 2012a). Thereby, molluscs are being considered as a sensitive and ecologically important group of invertebrates in the OECD test guideline programme.

In the present study, we observed a clear effect of the vertebrate EDC prochloraz on the reproduction of *P. antipodarum*, whereas the androgenic steroid trenbolone did not modulate the reproductive output of the snails at the tested concentrations. Both test guidelines with gastropods have limited ability to identify EDCs unequivocally, as the analysed endpoints refer to apical effects and do not prove that an endocrine-mediated pathway is responsible for the observed effects. Therefore, the reproduction test with *P. antipodarum* and *L. stagnalis* should not be treated as a surrogate for tests with vertebrates but as an addition to the existing OECD test battery for the risk assessment of chemicals.

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Conflict of interest

The authors declare to have no financial or non-financial conflict of interest.

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